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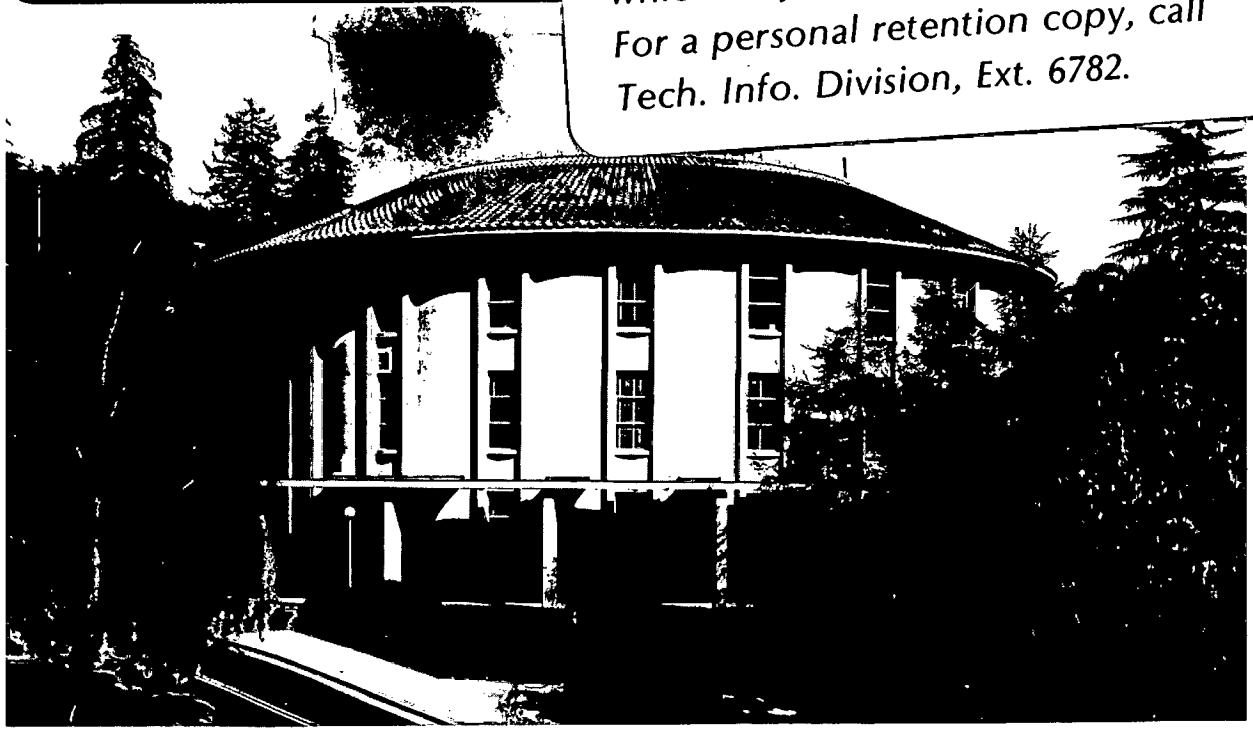

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TERPENOID BIOSYNTHESIS IN EUPHORBIA LATEX

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Key words: Euphorbia lathyris latex, triterpenol, triterpene esters, biosynthesis

SUMMARY

The latex of Euphorbia lathyris can utilize acetate, pyruvate and mevalonate for triterpene synthesis in vitro. Acetyl-CoA, HMG, HMG-CoA and IPP were not effective as precursors for triterpene biosynthesis. Acetate is utilized only by the terpenoid pathway and by the tricarboxylic (TCA) cycle; it is not used for fatty acid synthesis in this system. However, phospholipids were found to be efficient acyl donors for triterpene ester synthesis. The observed selectivity of precursor utilization as well as the observed rates for product formation indicate separate sites for triterpenol and triterpene ester synthesis and that one is not precursor for the other.

INTRODUCTION

Triterpenoids are the major constituents of many Euphorbia latexes [1]. These secondary metabolites can be stored in the latex as triterpenols or in the esterified form as acetates or fatty acid esters. As much as 50% of the latex dry weight can be composed of triterpenoids and their derivatives. In addition to terpenoids, latexes may contain polyisoprenes, protein, phospholipids and inorganics.

The role of the latex and the specialized laticifer cell in isoprenoid synthesis is still obscure. The theory that the latex serves as storage for compounds which the plant does not utilize has often been proposed. However, several of the triterpenoids stored in Euphorbia latexes are apparently not metabolic end products; specifically the conversion of cycloartenol (a common latex sterol) to phytosterols has been demonstrated in a number of higher plants [2].

Latex can be used as an enzyme system for terpenoid biosynthesis *in vitro*. Early work by Ponsinet and Ourisson has shown that both acetate and mevalonate are utilized for terpenoid biosynthesis in several Euphorbia latexes *in vitro* [3,4]. Bisboer and Mahlberg reported on the incorporation of mevalonate into the latex sterols of *E. tirucalli* [5]. Groenvelt *et al.* studied the *in vivo* synthesis of latex triterpenoids in defoliated stems of *E. lathyris* seedlings and in cotyledons [6,7]. The most extensively investigated system is the latex of *Hevea brasiliensis* which produces natural rubber. Rubber biosynthesis, however, is a special case of isoprenoid synthesis, both because of the high molecular weight of the product and because of the steroeochemical

requirement of cis configuration about the double bonds. Lower terpenoids are formed by the cyclization of trans prenyl pyrophosphates.

Pyruvate, acetate, acetoacetate, mevalonate, HMG-CoA and IPP all proved to be effective precursors of rubber in Hevea latex. HMG-CoA was reported to be the best substrate for rubber synthesis [8,9]. Hepper and Audley suggested that the reduction of HMG-CoA to MVA is the rate limiting step in rubber biosynthesis; this step is also considered important in the regulation of sterol biosynthesis in yeast and animals [9,10].

The major components of E. lathyris latex are tetracyclic triterpenoids which are stored as triterpenols and as the fatty acid esters of these triterpenols. These lipids constitute 50% of the dry latex weight. The triterpenols have been identified as cycloartenol, 24-methylene cycloartenol, lanosterol and an isomer of lanosterol [11]. Euphol is sometimes present in trace quantities. The relative amounts of the four major triterpenoids fluctuate from plant to plant; however, lanosterol is always the predominant component. The identical triterpenes are also found esterified to fatty acids. The fatty acid moieties have been identified as palmitic, dodecanoic, decanoic and 2,4-decadienoic [12]. Endogenous latex triterpenols predominate over the triterpene esters 2:1.

In addition to the triterpenoids we have isolated another major lipid class in E. lathyris latex, and identified these as phospholipids. The fatty acids obtained by hydrolysis of these phospholipics were identical to the ones isolated from the triterpene esters; the relative abundance of the various fatty acids was also similar for the two cases. Inorganics constitute approximately 8% of the dry latex weight. Ca^{+2} (7.5%) is the major component, Na^{+} and K^{+} are present in lesser amounts.

Protein is estimated to be 10%of the dry weight from nitrogen analysis and protein assays.

This paper reports on several aspects of terpenoid biosynthesis in E. lathyris latex. Various potential isoprenoid precursors were tested as substrates for terpenoid synthesis in this system. The kinetics for the formation of the two major classes of triterpenoids, the triterpenols and triterpene esters, are described.

MATERIALS AND METHODS

Materials.

Radiochemicals: [2-^{14}C] acetic acid, sodium salt (57.5 mCi/mmol); [1-^{14}C] isopentenyl pyrophosphate, ammonium salt (53 mCi/mol); D-[U-^{14}C] glucose (296 mCi/mmol); D-[U-$_{14}$C] glucose-6-phosphate, sodium salt (302 mCi/mol); 3-hydroxy-3-methyl[3-$_{14}$C] glutaric acid (56.6 mCi/mol); 3-hydroxy-3-methyl[3-$_{14}$C] glutarylCoA (56.6 mCi/mmol); [1-^{14}C]acetyl CoA (55 MCi/mol); L-α-phosphatidylcholine, di [1-^{14}C] palmitoyl (115 mCi/mmol), and L-lysophosphatidylcholine [1-^{14}C] palmitoyl (57 mCi/mmol) were purchased from Amersham Radiochemicals. [3-^{14}C] pyruvate, sodium salt (20 mCi/mmol); R-[5-^{3}H] mevalonic acid, triethylammonium salt (5.3 Ci/mmol); RS-[2-^{14}C] mevalonolactone (50 mCi/mmol); and [2-^{3}H] acetic acid, sodium salt (1.6 Ci/mmol) were obtained from New England Nuclear.

Latex Incubation.

Latex was obtained from 4 to 6 month old greenhouse propagated Euphorbia lathyris plants by cutting the leafstalks of full grown leaves. After cutting the petiole with a razor blade the expelled drops of latex were collected and gently mixed. The latex was divided into aliquots

immediately after collection. Typical incubations were done by using 200 µl of latex with 5 mM $MgCl_2$, 3 mM glutathione in all experiments, in addition to the radioactive precursor, cofactor or buffer. The final volume of the mixture was between 225 and 240 µl. The incubation was stopped by the addition of several ml methanol, and the latex was then taken to dryness under a stream of nitrogen. The solid residue was washed with 4 x 2 ml of water. The water solution was extracted with $CHCl_3$. The $CHCl_3$ layer was analyzed for phospholipids; the water layer contained protein and the tricarboxylic (TCA) cycle acids. The residue was extracted with 5 ml acetone by stirring for 12 hrs at ambient temperature. The acetone solution was withdrawn and the solid was rinsed with additional acetone. The combined extracts were concentrated to a small volume and analyzed for triterpenoids. The isolation and separation of the various latex constituents is outlined in Fig. 1.

Analytical Techniques.

Analysis of TCA acids. TCA cycle acids (fumarate, citrate, malate and succinate) were analyzed by High Pressure Liquid Chromatography (HPLC) on a 300 x 7.8 mm Aminex HPX-87H cation exchange column purchased from BioRad. The mobile phase was 0.025 N H_2SO_4; detection: UV at 210 nm, flow rate: 0.3 ml/min. The individual acids were collected and the radioactivity was determined by scintillation counting.

Analysis of triterpenoids. The triterpenols were separated from the triterpene esters on thick layer silica gel G plates (Analtech); eluted with ether:petroleum ether 3:1. Bands from the plates were scraped into vials and the radioactivity was measured in the gel phase, or eluted from the silica gel and analyzed further by reverse phase HPLC on 5 µ

ODS columns (Altex). Mobile phase CH_3OH:CH_3CN 90:10, detection UV at 210 nm, flow rate: 1 ml/min. Two 25 cm columns were coupled to achieve baseline resolution of the five triterpenols. The individual triterpenoids were collected and the radioactivity was determined by scintillation counting.

Hydrolysis of triterpene esters. Several mg of triterpene esters, isolated from a thick silica plate, were hydrolyzed with 10% KOH in MeOH for 12 hrs at ambient temperature. After addition of water and acidification the triterpenols and fatty acids were extracted with diethyl ether. The concentrated ether extract was applied to a basic silica gel plate (Analtech silica gel G with 0.1 N NaOH) and eluted with diethyl ether:petroleum ether 3:1. The radioactivity in the bands corresponding to the triterpenols and fatty acids was determined by scintillation counting.

Analysis of phospholipids. TLC of the $CHCl_3$ extract (Fig. 1) on silica gel G eluting with $CHCl_3$:MeOH:H_2O (65:25:4) showed three components when visualized with $(NH_4)_2MoO_4$-$HClO_4$(Hanes reagent). One of these components coeluted with dipalmitoyl phosphatidylcholine; the R_F of the other two bands indicated that these were lysocompounds. The phospholipids were hydrolyzed by the same method as the triterpene esters. The fatty acids were methylated with BF_3-methanol, and the methyl esters were analyzed by capillary gas chomatography on a 70 m x 0.2 mm ID OV-1 column at 200^o. The identities of the fatty acids were determined by coelution with standards and by analysis of mass spectra.

RESULTS AND DISCUSSION

Comparison of Different Precursors as Substrates for Terpenoid Biosynthesis

Several potential radiolabeled isoprenoid precursors were tested as substrates for triterpenoid synthesis in E. lathyris latex. The comparative efficiency of these substrates is shown in Table I. Pyruvate, acetate and mevalonate proved to be equally effective substrates in this system, but HMG and HMG-CoA were not utilized at all for terpenoid synthesis. The incorporation of acetyl-CoA and IPP was negligible. The inefficiency of these precursors may be attributed to permeability difficulties and thus poor transport to the site of terpenoid synthesis. The lack of incorporation of HMG-CoA is particularly interesting since this substrate was observed to be the most efficient in Hevea latex.

Our finding that mevalonate is transformed into labeled triterpenes is in contrast to the conclusions of Groenveld et al. [6] who concluded, based on results of experiments with defoliated stems of E. lathyris, that mevalonate is not utilized in latex triterpene synthesis. Furthermore, the observations made by the same authors that glucose is an efficient precursor of latex triterpenes is not in agreement with our results (Table I). The poor incorporation of glucose indicates that glycolysis is not a significant process in tapped latex. The negligible incorporation of glucose that we observed is probably due to contamination of the latex with leakage from the surrounding cells, an unavoidable circumstance of the tapping process. It is therefore very likely that in vivo glycolysis takes place in the wall lining cells of the laticifer, and a glycolytic end product, such as pyruvate, is transported to the laticifer for terpenoid biosynthesis.

Table I

Comparison of Precursors for Terpenoid Synthesis in E. lathyris Latex

Incorporation represents total terpenoid synthesis (triterpenols plus terpene esters). Incubations were done on 200 µl latex, with Mg^{+2} (5 mM), glutathione (3 mM) at pH 5 for 24 hrs. Maximum incorporation is 2 nmoles of carbon* in 24 hrs. Blank experiments were performed on boiled latex.

Precursor	Relative Incorp.	Precursor	Relative Incorp.
Glucose	4	HMG-CoA	0
Glucose-6-P	16	HMG	0
Pyruvate	100	MVA acid	100
Acetate	100	MVA lactone	1
Acetyl CoA	2	IPP	3

* Incorporation is calculated from the specific activity of the precursor. For the case of glucose and glucose-6-P the correction was made for carbon atoms not utilized in terpenoid biosynthesis.

Incubation of Radioactive Phospholipids

Neither the fatty acid moiety of the triterpene esters, nor the phospholipids are labeled when radioactive acetate or MVA are incubated in latex. Thus de novo fatty acid biosynthesis does not take place in latex in vitro.

The source of fatty acids for triterpene ester synthesis are phospholipids. When 1-[^{14}C]palmitoylphosphatidylcholine or di-[1-^{14}C]palmitoylphosphatidylcholine are incubated in latex, labeling of the fatty acid moiety of triterpene esters occurs. The triterpenols are not labeled. In 24 hrs a maximum of 40 nmol of palmitic acid is transferred from phospholipid to triterpene ester. The lyso compound is the superior acyl donor. Transfer from the di-palmitoyl compound is much less efficient; only 1 nmol of fatty acid transfer was observed.

Incubations of radioactive phospholipids in boiled latex resulted in a 99% reduction in the labeling of the triterpene esters, compared to the non-deactivated latex. The acyl transfer from phospholipid to triterpene esters is therefore an enzymatic process.

Effect of Cofactors and pH

The pH of E. lathyris latex, measured immediately after tapping, varies from 4.5 to 5. Ponsinet and Ourisson have reported that incorporation of acetate in E. heliscopia latex increased with pH, reaching a maximum at pH 7, and they commented on the anomaly of the inherent latex pH being different from the pH required for maximum synthesis [3]. In E. lathyris latex, however, we observed no increase in synthesis at pH 7 vs. pH 5. Above pH 7, even in the presence of reducing agents, the latex turns black and biosynthesis is totally inhibited.

No exogenous ATP or NADPH is needed for terpenoid synthesis in E. lathyris latex. Addition of either of these cofactors in various concentrations (3-10 mM) did not stimulate triterpenoid biosynthesis. An ATP generating system must therefore be present in the latex, but since glucose proved to be such a poor substrate, glycolysis is probably not the source of ATP. However, when acetate is used as a substrate, TCA cycle acids are labeled. In 24 hrs the TCA acids have 1/3 to 1/2 of the radioactivity contained in the terpenoid fraction. The observed TCA cycle activity indicates that mitochondria are the energy source of terpenoid biosynthesis in vitro.

Rates of Triterpenoid Synthesis

The rates of triterpenol and triterpene ester synthesis in a dual label experiment from 5-^{3}H-MVA and 2-^{14}C-acetate are shown in Fig. 2. Approximately 50% of the synthesis is completed within 6 hrs. In 24 hrs the radioactivity in triterpene esters is five times that in the triterpenols in this case; results of several other 20-24 hr incubations confirm that radioactivity of the triterpene esters is always higher than that of the triterpenols. The ratios vary from 10:1 to 5:1. The overall rate of synthesis of the triterpene esters from both acetate and mevalonate are considerably greater than those of the triterpenols. This pattern is reproducible; it is also observed in time course experiments with acetate only as the substrate. At the earliest time point shown in Fig. 2 (15 min) both the triterpenols and the triterpene esters are equally labeled when mevalonate is the substrate, whereas the incorporation into triterpene esters is already twice that of the triterpenols when acetate is the precursor. Further analysis of the triterpenol fraction by HPLC showed that all of the individual compounds that com-

prise this set are labeled at 15 min; the rates for all five triterpenols are linear between 15 min and 6 hrs with 24-methylene cycloartenol showing the fastest rate and euphol the slowest (data not shown).

To further elaborate the initial rates of terpenoid synthesis in this system, the incorporation of MVA and acetate up to 12 min was investigated. At these very short incubation times only ^{3}H-labeled substrates can be used, because even carrier free ^{14}C label is below detection limits. The initial rates of terpenoid synthesis from 5-^{3}H-MVA and 2-^{3}H-acetate are shown in Figs. 3 and 4, respectively. These experiments were done on different latex samples. Therefore, the data of Fig. 2 and Figs. 3 and 4 cannot be quantitatively compared because of variability of the latex itself. However, the 12 min labeling patterns shown in Figs. 3 and 4 agree well with the 15 min data points of Fig. 2. These sets of data are complementary, and together they describe latex terpenoid synthesis in 24 hrs, including the initial rates. As shown in Fig. 3, the rates of triterpenol and triterpene ester syntheses from MVA are equal (within experimental error); at the earliest time point in this experiment (30 sec) they are both labeled. When acetate is used as the precursor the rates are not equal (Fig. 4), but even in 15 sec both the triterpenols and the triterpene esters are labeled.

The behavior of the triterpene ester and triterpenol synthesis rates from both MVA and acetate indicates independent paths of formation for these two sets of compounds. Significantly, we do not observe any delay in the synthesis of either the triterpenols or their esters, which would be expected if either set served as the precursor of the other. The con-

clusion that triterpenol and triterpene ester synthesis take place independently is supported by experiments in which radioactive triterpenol or triterpene esters were reincubated in fresh latex. No interconversion of these occurred. However, the possibility cannot be ruled out that in the reincubation experiments the terpenoids did not reach the site of the putative esterification or ester hydrolysis.

Effect of Osmolality in Triterpenoid Synthesis

The results of our kinetic data indicate separate sites of synthesis for triterpenols and triterpene esters in this latex. The observed selectivity in precursor utilization implies that the transport of the precursor is an essential and important process in triterpenoid biosynthesis in this system. To date, no specific organelle (microbody) has been implicated in latex terpenoid synthesis, but we observed a marked decrease in biosynthesis on dilution of the latex. This effect of dilution as well as the effect of varying osmolality on terpenoid synthesis are shown in Fig. 5. Triterpene ester synthesis is significantly influenced by the change in osmolality whereas the triterpenol synthesis is affected to a lesser degree. Apparently the site of synthesis of triterpenols is not as osmotically sensitive.

CONCLUSION

Euphorbia lathyris latex utilizes isoprenoid precursors for triterpenoid biosynthesis selectively. Radioactive acetate is incorporated into the triterpenoids and the TCA acids, but not into the fatty acid moieties of triterpene esters and phsopholipids. Therefore, de novo fatty acid synthesis does not take place in E. lathyris latex in vitro. However, we have demonstrated that phospholipids are the acyl donors for

the triterpene esters. The observed kinetic behavior for the initial rates of triterpenol and triterpene ester formation indicates separate sites of synthesis for these two sets of compounds. The conclusion that triterpenol and triterpene ester biosynthesis are independent processes is supported by the observation that the initial rates do not show any delay for either set, which would be expected if one was the precursor of the other. In addition, the reincubation of radioactive triterpenols or triterpene ester did not result in their interconversion, indicating the esterification or ester hydrolysis is not the pathway for the formation of the triterpenols and the triterpene esters in this system.

The selectivity in precursor utilization shown in Table I indicates that transport substrate through a memgrane is an important aspect of terpenoid biosynthesis in E. lathyris latex. Electron micrograph studies of Hevea laticifers revealed the presence of mitochondria as well as two membrane-bound microbodies, the lutoids and "Frey-Wyssling" particles [13]. Whether either of these organelles has any function in terpenoid synthesis in Euphorbia latex is not known, but is under investigation. Our results of terpenoid synthesis in E. lathyris latex do indicate that this latex is not just a dilute cytoplasmic medium but it represents an organized system in which organelles may play an important role. Compartmentalization of terpenoid synthesis in latex is a reasonable model, and this may be an important feature of the regulatory mechanism of terpenoid synthesis in laticifer cells.

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FIGURE CAPTIONS

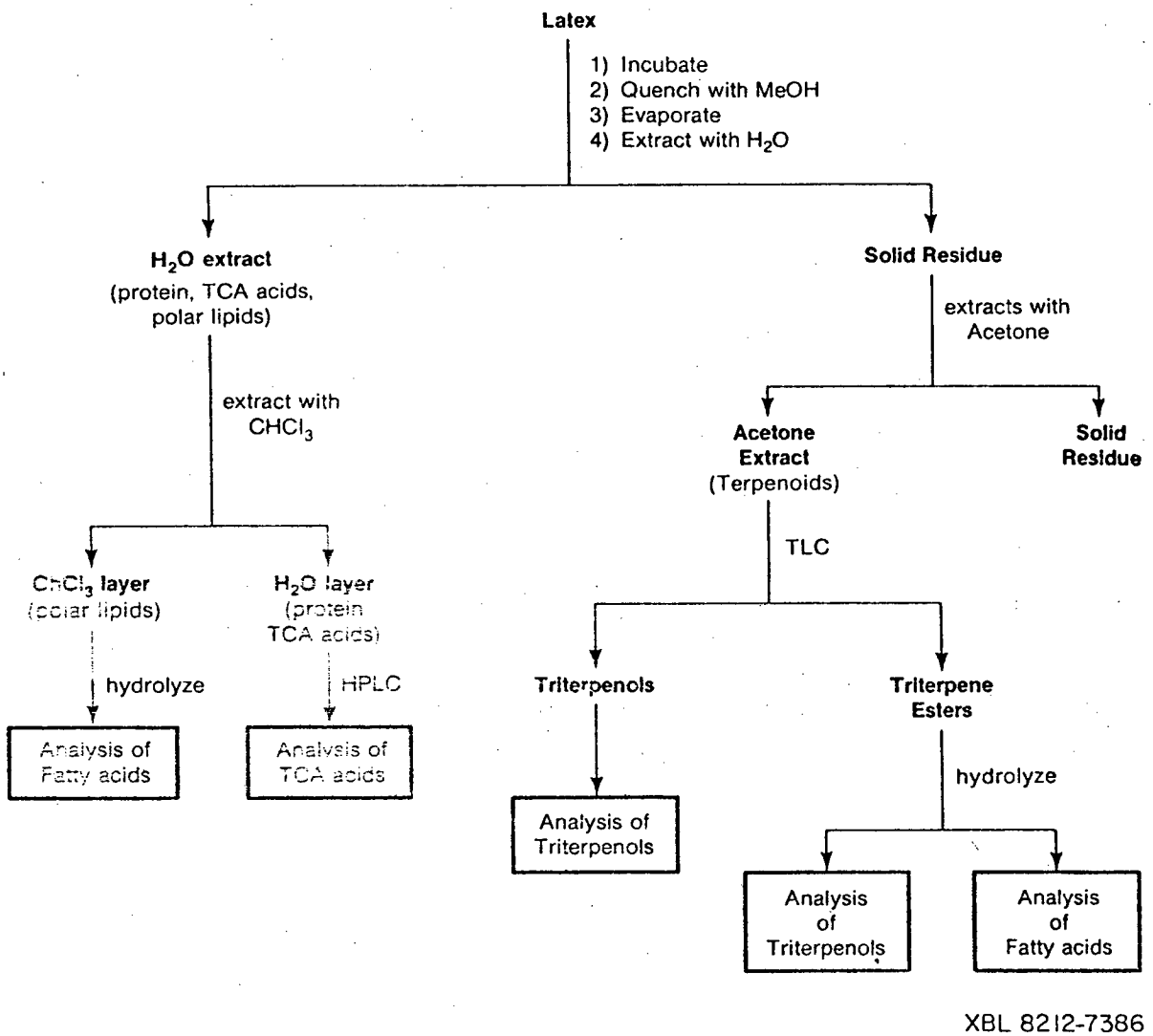
Fig. 1 Isolation and separation of Euphorbia lathyris latex constituents

Fig. 2 Time course incorporation of 5-^{3}H-MVA and 2-^{14}C-acetate into latex terpenoids _____ ^{14}C triterpenols, ____ ____ ^{3}H triterpenols, — —— —^{14}C triterpene esters, ... ^{3}H triterpene esters. Incubations were done on 175 µl aliquots of latex with glutathione (10 mM), $MgCl_2$ (4 mM), 30 µCi 5-^{3}H-MVA (5.3 Ci/mmole) and 3 µCi 2-^{14}C acetate (57 µCi/mmole).

Fig. 3 Incorporation of 5-^{3}H-MVA into latex terpenoids. □—□ triterpene esters, O—O triterpenols. Incubations were done under the same conditions as Fig. 2.

Fig. 4 Incorporation of 2-^{3}H-acetate into latex terpenoids. ●—● triterpenols, O—O triterpene esters. Incubations were done under the same conditions as in Fig. 2.

Fig. 5 Effect of dilution and changing osmolality on terpenoid synthesis from 2-^{3}H-acetate in Euphorbia lathyris latex. Acetate concentration: 0,37 mM (22 µCi), tenfold dilution.


Latex
1) Incubate
2) Quench with MeOH
3) Evaporate
4) Extract with H_2O
H_2O extract
(protein, TCA acids, polar lipids)
extract with $CHCl_3$
$CHCl_3$ layer
(polar lipids)
hydrolyze
Analysis of Fatty acids
H_2O layer
(protein TCA acids)
HPLC
Analysis of TCA acids
Solid Residue
extracts with Acetone
Acetone Extract
(Terpenoids)
Solid Residue
TLC
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hydrolyze
Analysis of Triterpenols
Analysis of Fatty acids
XBL 8212-7386

Fig. 1

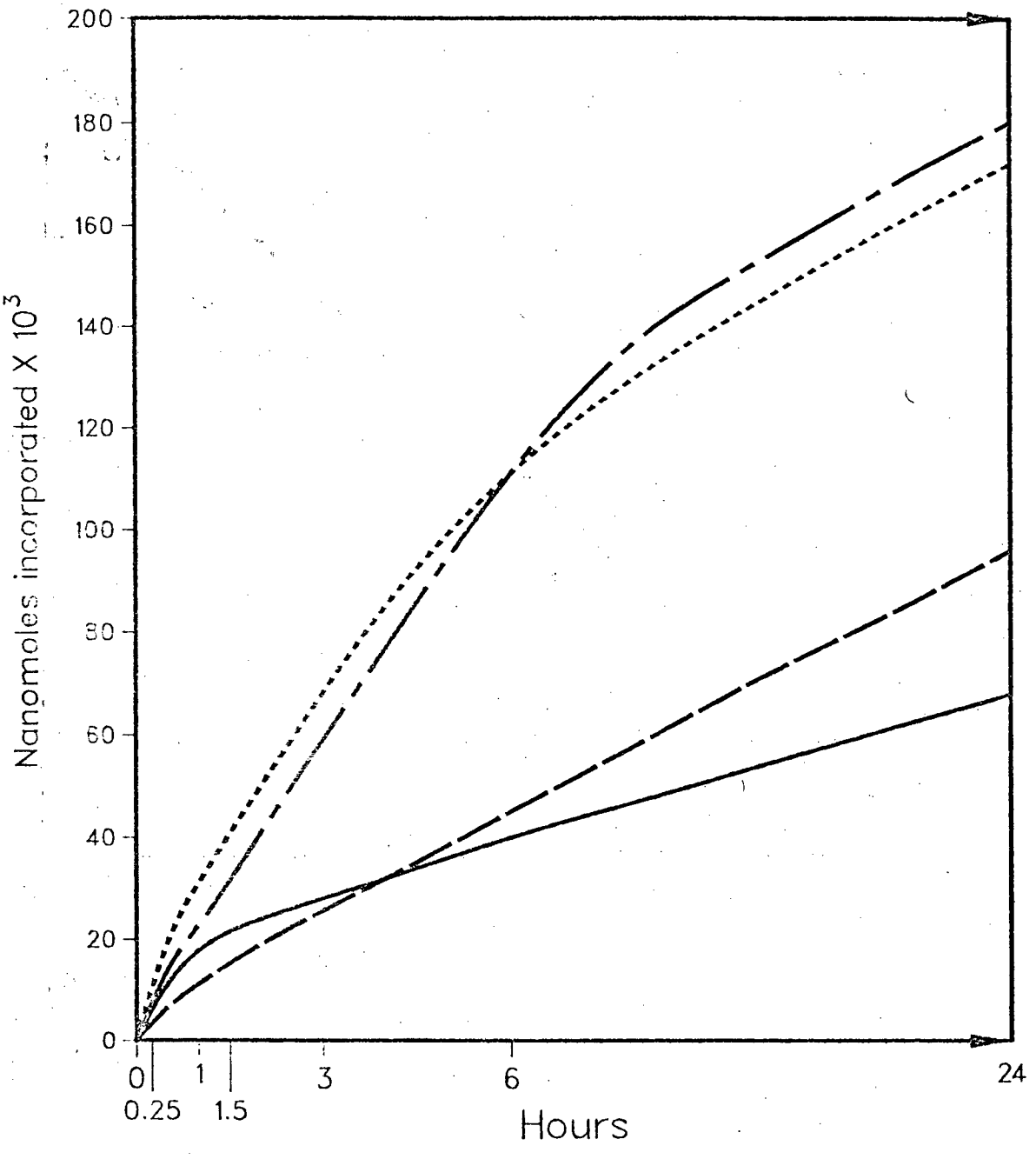

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Fig. 2

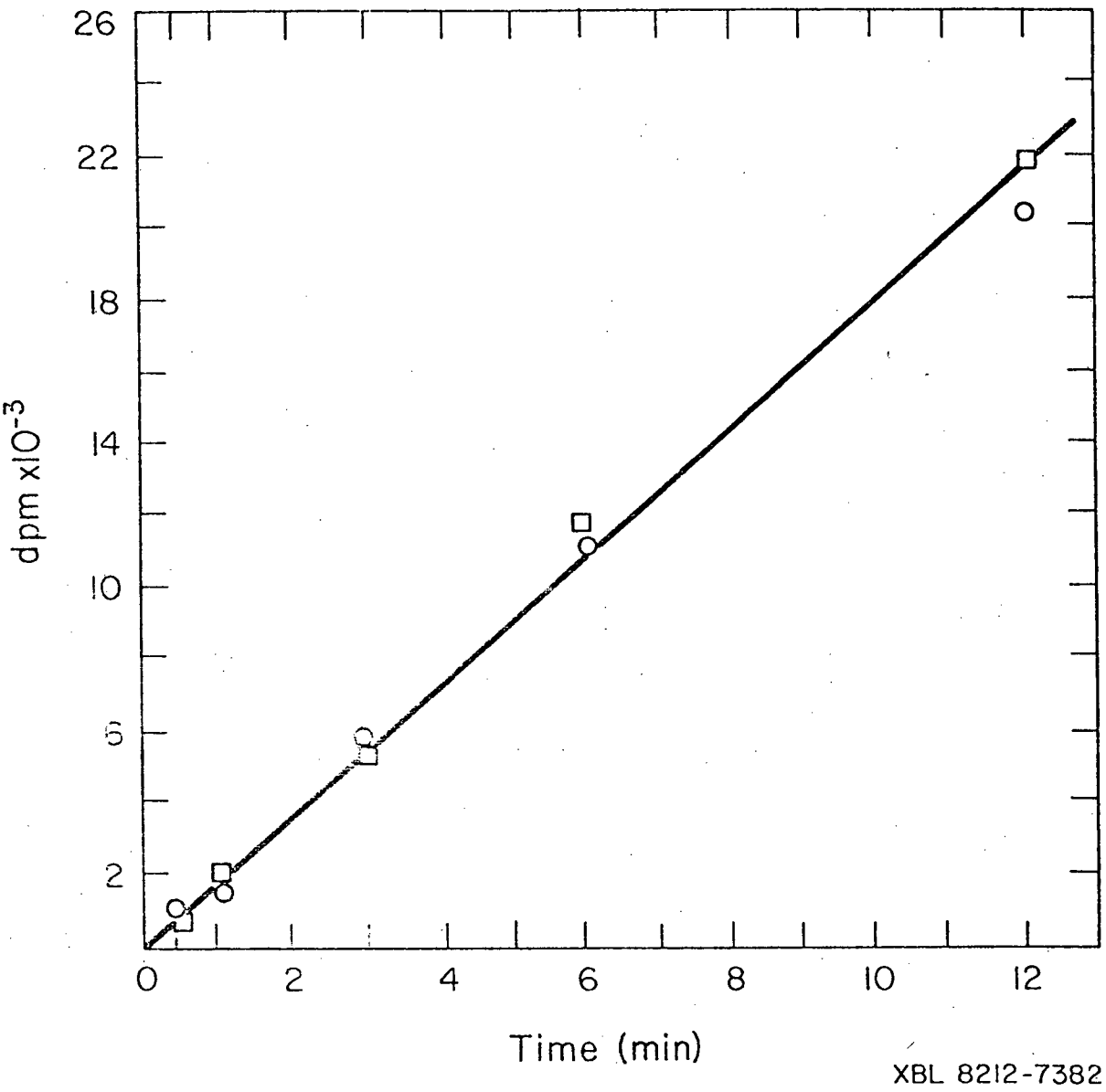

Fig. 3

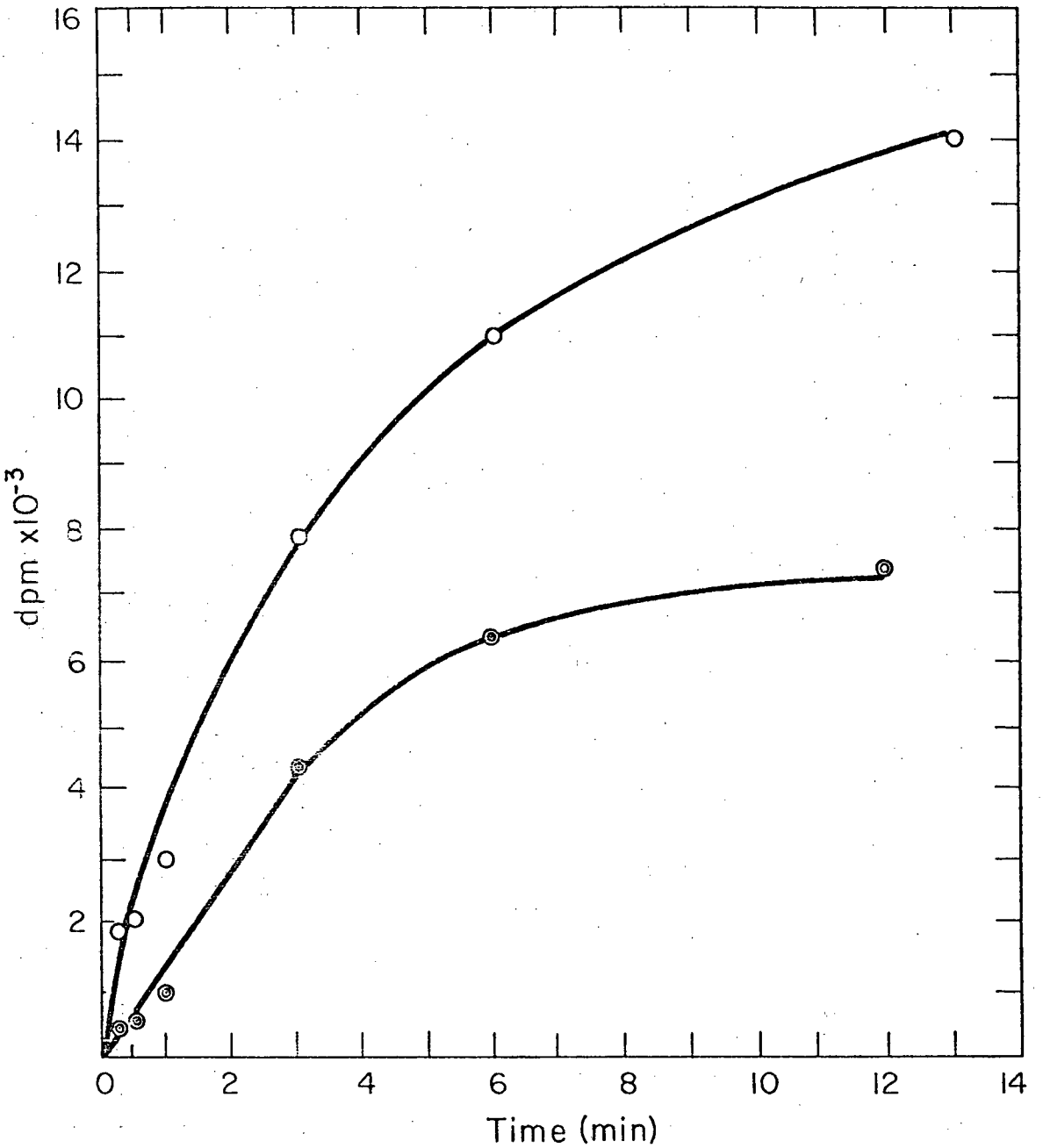

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Fig. 4

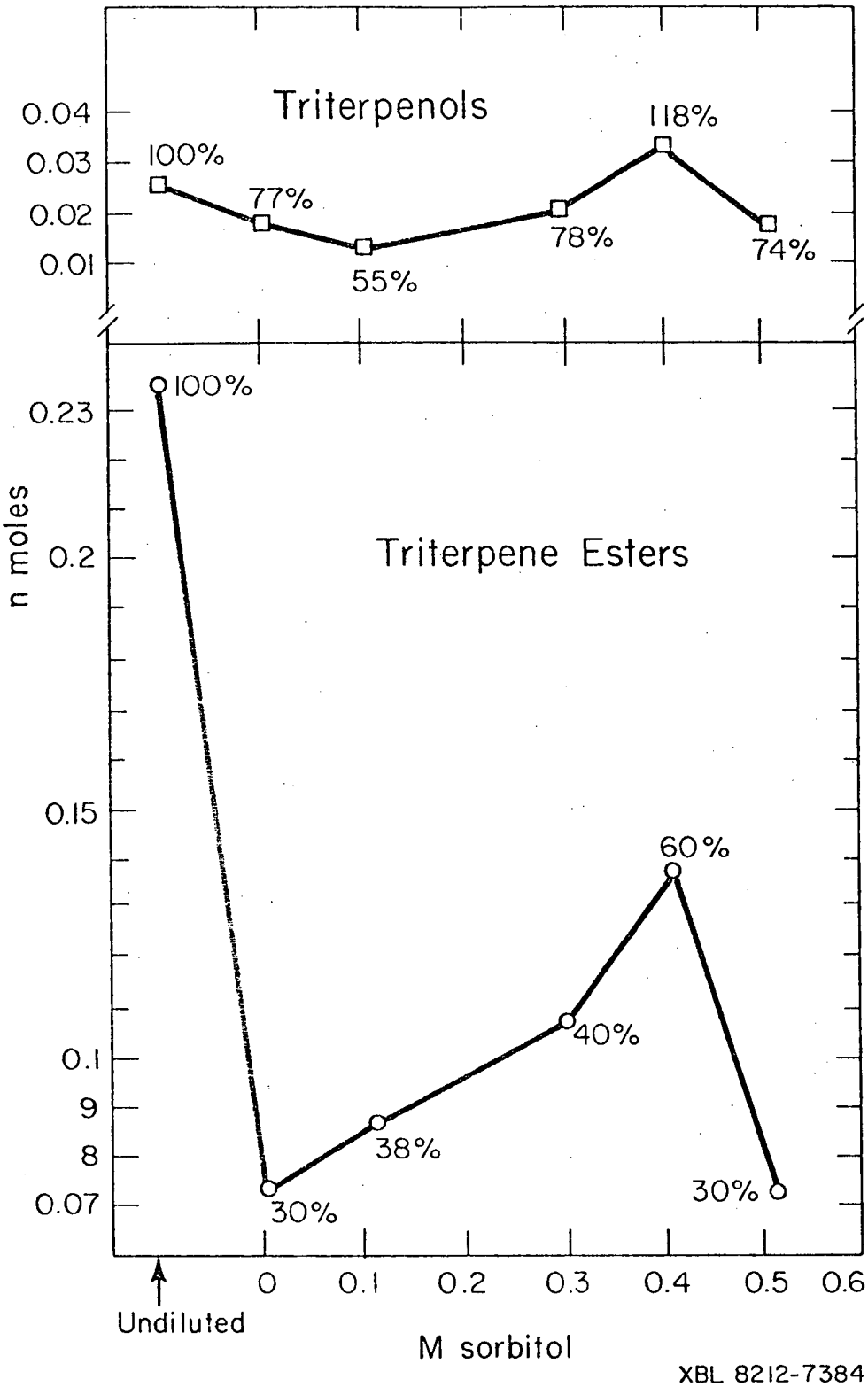
Triterpenols
0.04
0.03
0.02
0.01
100%
77%
55%
78%
118%
74%
n moles
0.23
0.2
0.15
0.1
9
8
0.07
Triterpene Esters
100%
30%
38%
40%
60%
30%
0
0.1
0.2
0.3
0.4
0.5
0.6
Undiluted
M sorbitol
XBL 8212-7384

Fig. 5

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